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Purification and Characterization of the Protein Kinase Encoded by the UL13 Gene of Herpes Simplex Virus Type 2

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The proteins encoded by the UL13 genes of herpes simplex virus types 1 (HSV-1) and 2 (HSV-2) have been predicted to be protein kinases. To identify the UL13 gene product, we have raised a rabbit polyclonal antiserum against a His · Tag-HSV-1 UL13 fusion protein. The antibody specifically reacted with the 60-kDa UL13 fusion protein expressed in *Escherichia coli* and also recognized 56- to 57-kDa late proteins in nuclear fractions of HSV-1- and HSV-2-infected cells. On the other hand, novel casein kinase activity was induced at the late stage of infection when Vero cells were infected with HSV-1 and HSV-2. The induction of the activity was most prominent in the nuclear fractions of HSV-2-infected cells and therefore we purified the protein kinase (PK) from the nuclear extracts by successive column chromatography (phosphocellulose, DEAE-cellulose, and hydroxyapatite) using casein as an exogenous substrate. The final preparation of the enzyme contained a single major protein with an apparent molecular weight of 56 kDa which was specifically reacted with the UL13 antiserum. The PK activity was optimal in the absence of NaCl and at relatively high pH. Acidic proteins such as casein and phosvitin were efficiently phosphorylated by the PK. A basic protein, protamine, which is the best substrate for the HSV-2 US3 PK, was not detectably phosphorylated but histone was a relatively good substrate for the UL13 PK. Phosphoamino acid analysis revealed that the PK phosphorylated serine and threonine but not tyrosine. Moreover the enzyme was found to be highly resistant to heparin, a potent inhibitor of casein kinase II (CK II) and also resistant to CK I-7, a synthetic inhibitor of CK I, but very sensitive to a bioflavonoid quercetin. These results indicate that the HSV-2 UL13 PK had unique catalytic properties different from those of cellular CK I, CK II, and the viral PK encoded by the US3 gene. We have also determined the complete nucleotide sequence of the HSV-2 UL13 gene. The overall amino acid homology between the HSV-2 and HSV-1 UL13 PKs was 85.9% and the homology was highly conserved in the C-terminal region.

INTRODUCTION

Herpes simplex virus (HSV) is a large DNA virus whose genome encodes at least 84 genes, and more than 10 virus-induced polypeptides have been shown to be associated with specific enzyme activities (Roizman and Sears, 1996). These include DNA polymerase (Powell and Purifoy, 1977; Knopf *et al.,* 1981; Nishiyama *et al.,* 1984), DNA helicase (Zhu and Weller, 1992), DNA primase (Crute *et al.,* 1989), thymidine kinase (Swain and Galloway, 1983), ribonucleotide reductase (Averett *et al.,* 1983; Goldstein and Weller, 1988), deoxyuridine triphosphatase (Preston and Fisher, 1984), uracil-DNA glycosylase (Caradonna *et al.,* 1987), and alkaline nuclease (Draper *et al.,* 1986; Shao *et al.,* 1993), all of which are synthesized as β polypeptides prior to viral DNA synthesis and are involved in DNA and deoxyribonucleotide metabolism. Furthermore, the virus induces enzymes catalyzing protein modification, such as protein kinase (PK) and protease (Welch *et al.,* 1991). At present, three open reading frames are shown to be associated with PK. The first of these genes to be identified, designated US3, was originally found to contain motifs conserved in eukaryotic PK by DNA sequencing analysis (McGeoch and Davison, 1986) and later the US3 gene product was shown to actually have PK activity (Frame *et al.,* 1987; Daikoku *et al.,* 1993). The US3 PKs of HSV types 1 and 2 have apparent molecular weights of 68 and 66 kDa, respectively. Both enzymes transfer phosphate from ATP to the seryl or threonyl residues in basic substrates such as protamine, but not in acidic substrates such as casein (Leader *et al.,* 1991; Daikoku *et al.,* 1993). The US3 PKs are not essential for viral replication in cell culture and for the establishment of latency, but essential for the maximum demonstration of neurovirulence (Purves *et al.,* 1987; Nishiyama *et al.,* 1992). Although the putative substrates of the US3 PKs have been identified (Purves *et al.,* 1991; Daikoku *et al.,* 1994, 1995), the targets and roles of this enzyme are still a matter for speculation.

The large subunit of HSV ribonucleotide reductase also possesses a PK activity (Chung *et al.,* 1989). This activity is located within the N-terminal 310 amino acids, a domain that is unique to HSV-1 and HSV-2 large subunits (Ali *et al.,* 1992). The large subunit of HSV ribonucleotide reductase is induced at immediate-early times

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postinfection prior to the synthesis of the small subunit, and therefore this PK is thought to play some role important for viral replication which is distinct from ribonucleotide reductase activity (Conner *et al.,* 1995).

The third gene, UL13, has been identified as a PK-related gene by Smith and Smith (1989) and Chee *et al.* (1989). This gene has counterparts not only in other alphaherpesviruses but also in beta- and gammaherpesviruses, while the US3 has counterparts only in alphaherpesviruses. The UL 13 product of HSV-1 has an apparent molecular weight of 55 to 57 kDa, is produced at the late phase of infection, and is incorporated into the virion as a structural component (Overton *et al.,* 1992). To investigate the role of the UL13 protein, recombinant viruses lacking UL13 have been constructed and the UL13 was found to be also dispensable for viral replication. Purves and Roizman (1992) have reported that UL13-defective mutants have different patterns of protein phosphorylation in infected cells from wild-type virus and that the UL13 product is responsible for the posttranslational processing associated with the phosphorylation of regulatory protein α22 (Purves *et al.,* 1993). On the other hand, Coulter *et al.* (1993) have shown that the UL13 protein is involved in phosphorylation of the virion tegument protein VP22. Moreover, Overton *et al.,* (1994) have reported that the UL13 product is necessary to produce the virion host shutoff effect mediated by the UL41 product. These observations indicate that the UL13 product play a role in the regulation of viral replication by means of the protein kinase activity. However, to the extent of our knowledge, there is no direct evidence that the protein possesses protein kinase activity. Cunningham *et al.* (1992) have shown that infection of cells induces a novel protein kinase activity which phosphorylates exogenous casein, but they have failed to purify the PK activity from the HSV-1-infected cells. Attempts have also been made to purify the UL13 protein from a cell line expressing UL13 and insect cells infected with recombinant-baculovirus, but they were unsuccessful (Cunningham *et al.,* 1992; Overton *et al.,* 1992). In this study, we purified the UL13 protein kinase from HSV-2-infected cells and characterized its biochemical properties.

MATERIALS AND METHODS

Cells and viruses

African green monkey kidney cells (Vero) were grown in Eagle's minimal essential medium (MEM) supplemented with 5% calf serum, 100 units of penicillin per milliliter and 100 μg of streptomycin per milliliter. Wild-type HSV-1 strain KOS and HSV-2 strain 186 were used and the virus stocks were prepared as described previously (Nishiyama *et al.,* 1984).

Chemicals and enzymes

Bovine milk α-, β-, and κ-caseins, histone (H-2), protamine, phosvitin, calmodulin, cAMP, spermine, *N*-ethylmaleimide, H-9, Staurosporin, quercetin, poly-L-lysine, poly-L-arginine, PMSF, ditiothreiotol (DTT), heparin, and other chemicals were purchased from Sigma Chemical Co. Phosphocellulose (P-11), DEAE-cellulose (DE52), and 3MM filters were from Whatman; [γ-^{32}P]ATP (6000 Ci/mmol) and Immobilon PVDF membrane were from Amersham International. Purified rat liver casein kinase I (CK I) and casein kinase II (CK II) were purchased from Promega and their purity is greater than 85% pure as determined by SDS–polyacrylamide gel electrophoresis (PAGE).

Construction of pET-28 expression vector

The UL13 gene of HSV-1 was amplified by polymerase chain reactions (PCR) using synthetic oligonucleotides primer-1 (CGACATATGGATGAGTCCCGCAGACAGC) and primer-2 (TCTGTCGACAGCGCGTGCCGCGCGCAC). *Nde*I and *Sal*I sites were incorporated into the primer-1 and primer-2, respectively, to facilitate cloning. The PCR consisted of an initial 2-min denaturation step at 94°, followed by 29 cycles of denaturation (94°, 1 min), annealing (56°, 2 min) and extension (72°, 3 min). The 30th cycle concluded with a 10-min extension step. Reaction mixture (100 μl) contained 0.1 μg of HSV DNA, 1 μM of primer-1 and primer-2, 2.5 U of PFU DNA polymerase, 0.2 m*M* each of deoxyribonucleoside triphosphate, 10% DMSO, 20 m*M* Tris–HCl (pH 8.8), 2 m*M* $MgSO_4$, 10 m*M* KCl, 10 m*M* $(NH_4)_2SO_4$, 0.1% Triton X-100, and 0.1 mg/ml BSA. The PCR product (1565 bp) was isolated, digested with *Nde*I and *Sal*I, and cloned in frame into the downstream of the region encoding the initiating ATG plus six histidine (6xHis) residues in a *Escherichia coli* expression vector pET-28b (Novagen, New York) to give pET-28 UL13.

Preparation of polyclonal antiserum against the UL13 product

Plasmid pET-28 UL13 was transformed into *E. coli* strain BL21 (DE3) which, following induction with IPTG, expressed high levels of 6xHis-tagged UL13 fusion protein. The UL13 fusion protein was solubilized and purified by means of an affinity column (His · Bind resin, Novagen, New York) according to the protocol supplied by the manufacture. Rabbits were subcutaneously injected with 200 μg of the purified UL13 fusion protein which was emulsified with complete Freund's adjuvant. Four weeks after the first injection, the rabbits were given two booster injections at intervals of 2 weeks. Ten days after the last boost, the rabbits were bled and antisera were prepared.

Assay of protein kinase activity

The standard assay mixture for the HSV UL13 PK contained, in a total volume of 50 μl, 10 m*M* Tris–HCl (pH 8.0), 10 m*M* $MgCl_2$, 1 m*M* DTT, 0.1 m*M* [γ-^{32}P]ATP (300

cpm/pmol), and casein (2 mg/ml). The assays for CK I and CK II were carried out according to the protocols from the suppliers. The reaction mixture for CK I contained 25 m*M* Tris–HCl (pH 7.4), 10 m*M* $MgCl_2$, 0.1 m*M* [γ-^{32}P]ATP (300 cpm/pmol), and casein (2 mg/ml). For the assay of CK II, 200 m*M* NaCl was added to the above mixture. The reaction was started by the addition of enzyme. After incubation at 37° for 15 min, aliquots of the mixtures were withdrawn and spotted onto Whatman 3MM filter discs. The filter discs were washed twice for 15 min in 10% (W/V) trichloroacetic acid (TCA), three times in 2% TCA, once in absolute ethanol, and then dried. The radioactivity was measured by liquid scintillation spectrometry.

Phosphoamino acid analysis

Casein was phosphorylated by the purified HSV-2 UL13 PK in the standard assay mixture. The radioactive casein was precipitated by addition of 10% TCA, acid hydrolyzed, analyzed by thin-layer electrophoresis, and visualized by autoradiography, as described previously (NG *et al.,* 1994). Unlabeled phosphoserine, phosphothreonine, and phosphotyrosine was used as standards and visualized with 0.2% ninhydrin.

Extraction and purification procedures

Confluent monolayers of Vero cells were infected with virus at a multiplicity of 5 PFU/cell, incubated at 37° for 20 hr, and harvested after washing twice with phosphate-buffered saline (PBS). The cells were pelleted by centrifugation at 1000 rpm for 10 min and resuspended in RSB buffer (10 m*M* Tris–HCl, 10 m*M* NaCl, 1.5 m*M* $MgCl_2$, pH 7.4) and left for 5 min on ice. After adding NP-40 to a final concentration of 1%, the suspension was gently mixed by vortex and separated into the nuclear and cytoplasmic fractions by low-speed centrifugation (1500 rpm, 10 min). The nuclear fractions were then resuspended in 40 ml of buffer A (20 m*M* Tris–HCl, 1 m*M* EDTA, 10 m*M* 2-ME, 1 m*M* PMSF, 10 μg/ml aprotinin, 10% glycerol, pH 7.5) containing 400 m*M* NaCl and 1% NP-40, and sonicated for 30 sec six times in ice. Extracts were centrifuged for 30 min at 15000 *g* at 4° and the supernatant was dialyzed against buffer A and applied to preequilibrated phosphocellulose column (25 $\times$ 100 mm). The column was washed with buffer A containing 50 m*M* NaCl and proteins were eluted with a linear gradient of 50 m*M* to 1 *M* NaCl in the same buffer. Fractions were collected and assayed immediately. Peak fractions from phosphocellulose column chromatography were dialyzed against buffer A and applied onto a DEAE-cellulose column preequilibrated with buffer A. The column was washed with 400 ml of buffer A and proteins were eluted with 400 ml of a linear gradient of 50 m*M* to 1 *M* in buffer A. Peak fractions from DEAE-cellulose column chromatography were dialyzed against buffer B (10 m*M* potassium phosphate (pH 7.5), 100 m*M* NaCl) and applied onto a hydroxyapatite column (1 $\times$ 6 cm) preequilibrated with buffer B. The column was washed with 50 ml of buffer B and proteins were eluted with 50 ml of a linear gradient of 10 m*M* to 1 *M* potassium phosphate (pH 7.5) containing 100 m*M* NaCl.

Western blotting

Cells were washed three times with PBS and lysed with 2$\times$ SDS sample buffer (125 m*M* Tris–HCl, 10% glycerol, 10% SDS, 5% 2-ME, pH 6.8). Then proteins were separated by electrophoresis in 10% polyacrylamide gels in the presence of 0.1% SDS. The separated proteins were electrophoretically transferred to an Immobilon PVDF transfer membrane. Nonspecific protein binding was blocked by treating the membrane with Tris-buffered saline (TBS; 25 m*M* Tris–HCl, 150 m*M* NaCl, pH 7.4) containing 3% BSA and 0.05% Tween 20 at 4° overnight. The membrane was washed once with TBS and incubated with anti-UL13 rabbit serum diluted 300-fold in TBS containing 0.1% BSA and 0.05% Tween 20 (TBS-T) at 37° for 1 hr. After washing three times with TBS-T, the membrane was incubated with horse radish peroxidase (HRP)-conjugated anti-rabbit immunoglobulin at 37° for 1 hr. The membrane was then washed three times with TBS-T and once with TBS, treated with ECL Western blotting detection system (Amersham Japan Co.), and exposed to Hyperfilm-ECL (Amersham Japan Co.)

Sequencing of the HSV-2 UL13 open reading frame

The UL13 gene of HSV-2 was sequenced by the primer extension/dideoxy chain termination method using ABI PRISM dye terminator cycle sequencing ready reaction kit (Perkin–Elmer). A 20.7-kb *Hin*dIIIB fragment of HSV-2 strain 186 DNA has been cloned into the *Hin*dIII site of plasmid pACYC 184 (Tsurumi *et al.,* 1986). The about 5.5-kb fragment, which contains the complete open reading frames of the UL13, UL14, UL15, and UL16 genes of HSV-2, was obtained by digesting the isolated *Hin*dIIIB fragment with *Bam*HI and *Eco*RI, and was inserted into the multicloning site of plasmid pUC19. Primers specific for the plasmid were used to initiate sequencing. Subsequent primer sequences were selected from the newly determined sequences. Using a total of 12 primers, 18 to 21 nucleotides in length, nucleotide sequences spanning the entire UL13 gene were obtained. Sequences were determined from three clones on both DNA strands. The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases with the following Accession Number AB001417.

RESULTS

Preparation of anti-UL13 product antiserum

As a first step toward the purification of UL13 PK, a rabbit polyclonal antiserum specific for this protein was

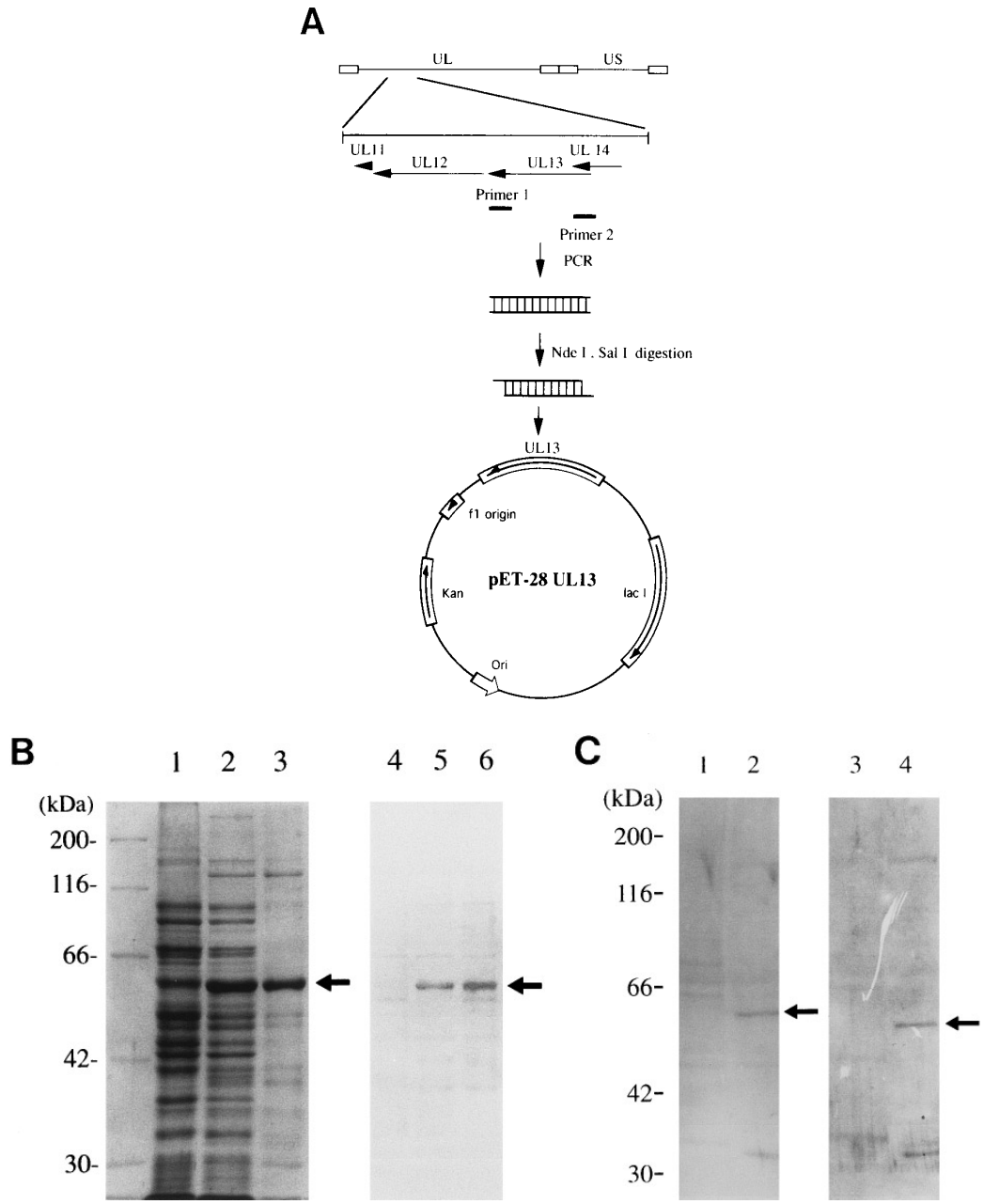

FIG. 1. (A) Schematic representation of the HSV genome containing the UL13 gene and strategy for the construction of the pET28 UL13. (B) Induction of the His · Tag-UL13 fusion protein in *E. coli* and its detection by Western blotting. *E. coli* harboring plasmid pET28-UL13 was grown in the absence (lanes 1 and 4) or presence (lanes 2 and 5) of IPTG. The His · Tag-UL13 fusion protein was purified by means of an affinity column (lanes 3 and 6). Proteins were separated by SDS–PAGE and stained with Coomasie blue (lanes 1, 2, and 3). Samples were also probed with the anti-UL13 antiserum (lanes 4, 5, and 6). (C) Detection of the UL13 gene product in infected cells by Western blotting. (A) Vero cells were mock-infected (lanes 1 and 3) or infected with HSV-1 (lane 2) and HSV-2 (lane 4) and harvested at 18 hr postinfection. Nuclear fractions of mock-infected or HSV-infected cells were analyzed by Western blotting using rabbit polyclonal antibodies against the His · Tag-UL13 fusion protein.

raised using antigen a recombinant His · Tag UL13 fusion protein. For this purpose the HSV-1 UL 13 gene was amplified by PCR using primers with either restriction endonuclease site of *Nde*I or *Sal*I. The amplified DNA was digested with *Nde*I and *Sal*I and cloned into the pET 28b expression vector under the control of the T7 RNA polymerase promoter. The resulting plasmid pET UL13 contained the complete sequence of the HSV-1 UL13 ORF fused in frame at the 3′ end of His · Tag sequence. A diagram of the cloning scheme is shown in Fig. 1A. High levels of the His · Tag-UL 13 fusion protein were expressed in *E. coli* BL21 (DE3) following induction by IPTG (Fig. 1B). The protein was solubilized by sonication in the presence of 1% NP-40, purified by a nickel-affinity column, and used for the production of anti-UL13 antiserum in rabbits.

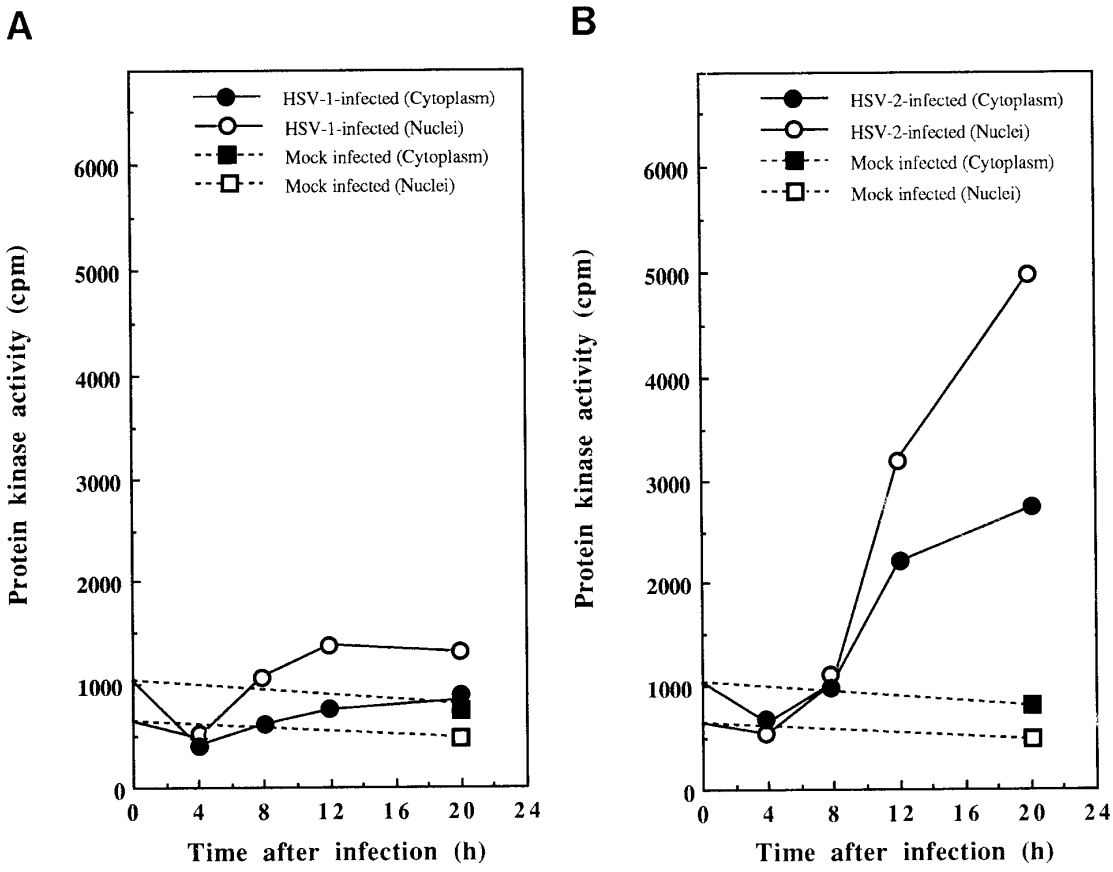

FIG. 2. Induction of protein kinase activity in infected cells. Vero cells were mock-infected or infected with HSV-1 (A) or HSV-2 (B) at a multiplicity of 5 PFU/cell and harvested at the indicated times after infection. The cells were separated into cytoplasmic (●, ■) and nuclear (○, □) fractions. Protein kinase activity of each fraction was measured by monitoring incorporation of ^{32}P from [γ-^{32}P]ATP into TCA-precipitable materials under the conditions described under Materials and Methods. Casein was used as a exogenous substrate.

To examine the reactivity and specificity of the anti-UL13 antiserum, Western blot analysis was performed. Vero cells were mock-infected or infected with HSV-1 or HSV-2 at a multiplicity of 5 PFU/cell and incubated for 18 hr at 37°. The nuclear fractions of the mock-infected and infected cells were prepared and the proteins of the fractions were electrophoretically separated on polyacrylamide gels, transferred to Immobilon PVDF membranes, and subjected to the Western blotting detection system using the anti-UL13 antiserum. As shown in Fig. 1C, the antiserum predominantly reacted with a polypeptide with an apparent molecular weight of 57 kDa which was present in nuclear extracts of HSV-1-infected cells but not in those of mock-infected cells. The antiserum could also react with a 56-kDa protein of HSV-2-infected cells. Time course experiments showed that the 57-kDa protein was detected after 8 hr postinfection, and that the intensity of the band increased gradually until 24 hr postinfection (data not shown). These results are consistent with those reported by Cunningham *et al.* (1992), supporting that the rabbit antiserum recognized the UL13 proteins induced by HSV-1 and HSV-2.

TABLE 1

Purification of HSV-2 UL13 Protein Kinase

	Volume (ml)	Protein (mg)[a]	Activity (pmol/min)	Specific activity (pmol/min mg)	Purification (-fold)
Nuclear extract	320	1360	74764	55.0	1
Phosphocellulose	84	8.4	10994	1308.8	23.8
DEAE cellulose	18	3.6	6033	1675.8	30.5
Hydroxyapatite	7	0.25	1833	7332.0	133.3

[a] Protein concentration was estimated using Bradford protein assay kits (Bio-Rad) with BSA as the standard.

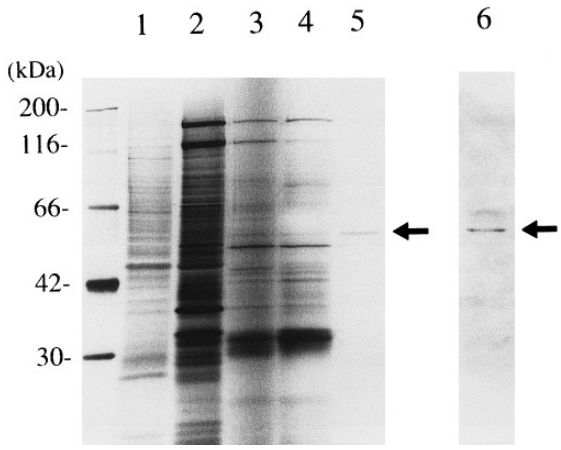

FIG. 3. Purification and identification of the UL13 PK of HSV-2. Uninfected (lane 1) and infected (lane 2) Vero cells were separated into cytoplasmic and nuclear (lane 1 and 2) fractions. Nuclear extracts of HSV-infected cells were subjected to successive chromatography on phophocellulose (lane 3), DEAE-cellulose (lane 4), and hydroxyapatite (lane 5), as described under Materials and Methods. The peak fractions of each chromatography were applied to SDS–PAGE, and the gels were stained with silver. The final preparation was analyzed by Western blotting using rabbit polyclonal antibodies against the His · Tag-UL13 fusion protein (lane 6).

Induction of a nuclear PK by HSV-1 and HSV-2

Induction of protein kinase activity was studied in nuclear and cytoplasmic extracts of mock-, HSV-1-, or HSV-2-infected cells, using casein as a exogenous substrate. Confluent monolayers of Vero cells were infected with each virus, incubated at 37°, and harvested for the assay of protein kinase activity at various times after infection. High levels of casein kinase activity were induced in the nuclei of HSV-2-infected cells and also a considerable increase of activity was observed in the cytoplasmic fraction (Fig. 2). Whereas, in HSV-1-infected cells the induction of casein kinase activity was not so conspicuous as that observed with HSV-2. The level of the casein kinase activity in the nuclear extracts of mock-infected cells was slightly higher than the background and remained approximately constant. The Western blot analysis shown in Fig. 1C suggests that similar amounts of UL13 protein were produced in HSV-1- and HSV-2-infected cells at 18 hr postinfection. However, the level of casein kinase activity induced in HSV-1- and HSV-2-infected cells differed by approximately fivefold at 20 hr postinfection. Although the reason is not clear yet, this might be due to different substrate specificity and optimal conditions of reaction.

In the above experiments, the casein kinase activity was assayed in the absence of NaCl, and similar patterns of the induction were obtained even when assayed in the presence of 500 m*M* NaCl, although the activity was much lower at this concentration of NaCl (data not shown).

Purification of the UL13 PK

Based on the above observations, we decided to purify the UL13 PK from the nuclear extracts of Vero cells infected for 20 hr with HSV-2. The nuclear extracts were subjected to phosphocellulose column chromatography. With casein as a substrate, one major peak with a minor second peak of protein kinase activity was observed in the elution profile from the nuclear extracts (data not shown). The major peak was eluted at 400 m*M* NaCl and was found to contain the 56-kDa protein which reacted with the anti-UL13 antiserum. Thus the first major peak fractions of casein kinase activity were further subjected to sequential column chromatography on DEAE-cellulose and hydroxyapatite. In both DEAE-cellulose and hydroxyapatite chromatography, we could observe a single major peak of casein kinase activity, which contained the 56-kDa protein. The whole steps of purification are summarized in Table 1. The nuclear casein kinase was purified approximately 130-fold in specific activity from the nu-

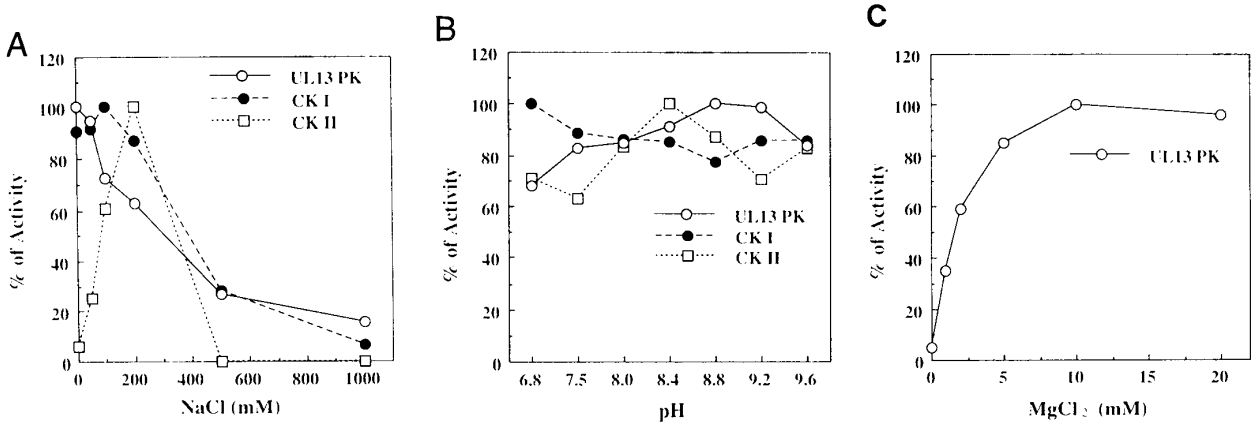

FIG. 4. Effect of NaCl (A), pH (B), and $MgCl_2$ (C) on the activity of the UL13 PK. The enzyme preparation was assayed for protein kinase activity with casein as substrate as described under Materials and Methods, except that the pH and the concentrations of NaCl and $MgCl_2$ were varied as indicated. The activity is expressed as percentage of the maximum values.

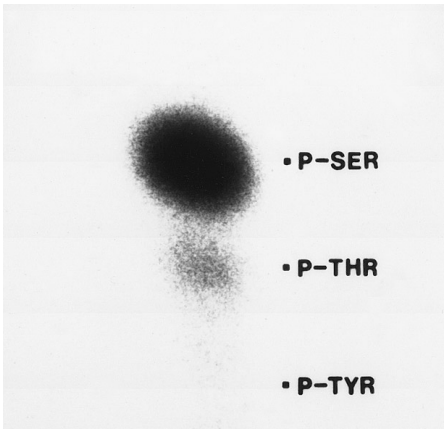

FIG. 5. Phosphoamino acid analysis of phosphorylated casein. Casein was phosphorylated *in vitro* by the purified HSV-2 UL13 PK and subjected to phosphoamino acid analysis as described under Materials and Methods. Unlabeled phosphoserine (P-SER), phosphothreonine (P-THR), and phosphotyrosine (P-TYR) were used as markers.

clear extracts of HSV-2-infected cells. The final preparation of the purified protein kinase contained one major protein of 56 kDa, which reacted with the anti-UL13 antiserum (Fig. 3).

Catalytic properties of the HSV-2 UL13 PK

The UL13 PK of HSV-2 was first characterized with respect to salt dependence, using casein as a substrate (Fig. 4A). Cellular CK I and CK II were used for comparison. The purified UL13 PK was sensitive to salt and was most active in the absence of NaCl. At 500 m*M* NaCl the activity was inhibited by about 70%. In contrast, The activity of CK II was strikingly stimulated by the addition of NaCl and was optimal at 200 m*M,* but in the presence of 500 m*M* NaCl almost no activity was detectable. The CK I activity was also stimulated by 200 m*M* NaCl but to a much lesser extent. At 500 m*M,* the CK I, like the UL13 PK, was inhibited by 70%. The pattern of salt dependence of the UL13 PK was similar to that of CK I rather than that of CK II. All of three PKs were active over a broad pH range, but the optimal pH was different (Fig. 4B). The optimal pH of the UL13 PK, CK I, and CK II was around pH 9.0, 6.8, and 8.4, respectively. The UL13 PK, like other protein kinases, had an absolute requirement for divalent cations. The optimum concentration of Mg^{2+} was between 10 and 20 m*M* (Fig. 4C).

To determine which amino acid residues of casein were phosphorylated by the UL13 PK, reaction was carried out under the optimal conditions of the enzyme, and phosphorylated casein was analyzed by phosphoamino acid analysis (Fig.5). The casein was found to be mainly phosphorylated at serine residues with a minor degree of phosphorylation at threonine residues, but there was no detectable phosphorylation at s tyrosine residue.

We next characterized the UL13 PK on substrate specificity (Table 2). The HSV-2 UL13 PK efficiently phophorylated acidic proteins, casein and phosvitin, but did not detectably phosphorylate a basic protein protamine which is the best substrate for the HSV-2 US3 PK. However, histone, one of basic proteins, was a relatively good substrate for the UL13 PK. Among α-, β-, and κ-caseins, β-casein was most efficiently phosphorylated by the UL13 PK. The substrate specificity of the HSV-2 UL13 PK was also similar to CK I rather than CK II.

Effects of inhibitors and activators on the activity of the HSV-2 UL13 PK

The effects of various inhibitors and activators on protein kinase activities were examined in the standard assay using casein as a substrate (Fig. 6). The HSV-2 UL13 PK was highly resistant to heparin, a potent inhibitor of CK II, the activity of which was completely inhibited at 5 μg/ml (Fig. 6A). Approximately 80% of the UL13 PK activity was retained even at 100 mg/ml heparin. At this concentration the activity of CK I was inhibited by 60%. The UL13 PK also showed a relatively high resistance to CKI-7 (Fig. 6B), a synthetic inhibitor of CK I (Chijiwa *et al.,* 1989). Interestingly the UL13 PK, like the HSV-2 US3 PK (Daikoku *et al.,* 1993), was very sensitive to quercetin, a bioflavonoid; only 2 μM quercetin was required for 50% inhibition (Fig. 6C). Both CK I and CK II were much more resistant to this compound.

The effects of other compounds are summarized in Table 3. The UL13 PK activity was slightly stimulated by the addition of spermine and protamine, but no enhancement was observed by the addition of cAMP and $CaCl_2$. Polyarginine has been shown to inhibit the HSV-2 US3 PK; at 200 μg/ml more than 50% of the activity is inhibited. However, polyarginine and polylysine exhibited no or only slight inhibitory effect on the activity of the UL13 PK and CK I, while both enhanced the activity of CK II. *N*-ethylmaleimide, a SH group blocker, inhibited the enzyme activity, indicating that the SH group plays a role in the phosphate transfer reaction of the UL13 PK. Sphin-

TABLE 2

Substrate Specificity of HSV-2 UL13 Protein Kinase, Protein Kinase CK I, and CK II

	Percentage of activity		
	UL13 PK	CK I	CK II
Casein	100	100	100
α-Casein	52	43	<3
β-Casein	118	129	68
κ-Casein	79	167	37
Histone	86	85	16
Protamine	<3	<3	<3
Phosvitin	63	94	30

Note. Reaction conditions are given under Materials and Methods.

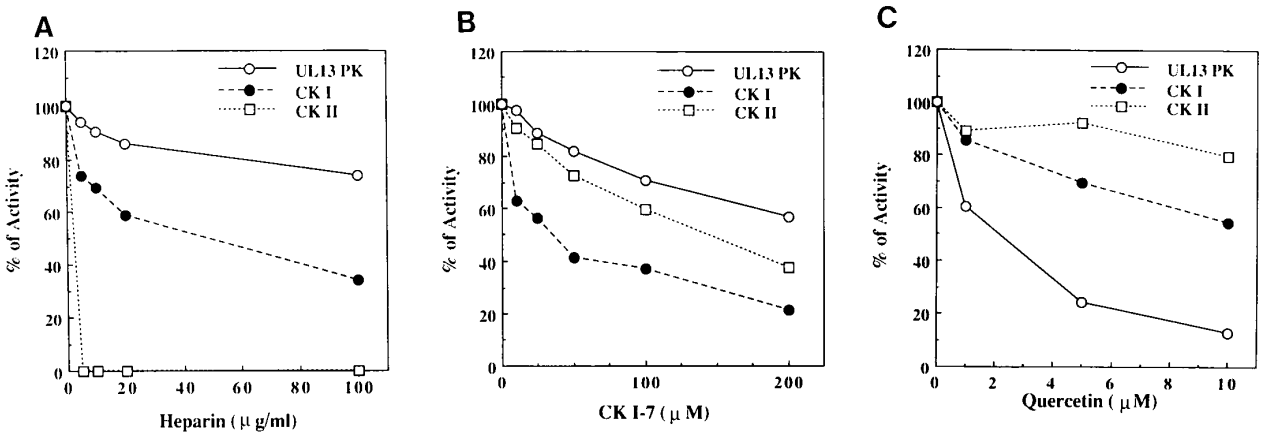

FIG. 6. Effect of heparin (A), CKI-7 (B), and quercetin (C) on the activity of the UL13 PK. Reactions were performed using casein as substrate as described under Materials and Methods, except that the inhibitors were added at the concentrations indicated.

gosine, an inhibitor of protein kinase C, did not show any significant inhibitory effect, but H-9, a potent inhibitor of cyclic nucleotide-dependent PKs, and staurosporin, a potent inhibitor of protein kinase C and cyclic nucleotide-dependent kinases, moderately inhibited the UL13 PK at relatively high concentrations of these inhibitors. Moreover, the incorporation of [γ-^{32}P] into the substrate was found to be suppressed by the addition of ATP but not GTP, suggesting that the UL13 PK does not utilize GTP as a phosphate donor.

Amino acids sequence of the HSV-2 UL13 PK

We have determined the nucleotide sequence of the UL13 region of the genome from a plasmid clone containing a *Hin*dIII B fragment of HSV-2 strain 186 inserted into pACYC184 (Tsurumi *et al.,* 1986), as described under Materials and Methods. Figure 7 presents the amino acid sequences in the one-letter notation of the polypeptide predicted for the HSV-2 UL13 PK. The UL13 protein of HSV-2 consisted of 518 amino acids and had a deduced molecular weight of 57,045 in agreement with the apparent molecular weight of 56 kDa for the purified enzyme. The calculated isoelectric point was 10.68 and slightly higher than that of the HSV-1 UL13. The overall amino acid homology between the HSV-1 and HSV-2 UL13 PKs was 85.9%. The homology was highly conserved in the C-terminal region of the UL13 protein; the homology of the C-terminal 150 amino acids was 92%, while that of the N-terminal 150 amino acids was 73.3%. The 47 amino acids between residues 427 and 483 were completely identical to each other. When the predicted amino acid sequences were compared with those of other PK family members, major subdomains which are conserved in various PKs could be readily identified, but a few subdomains were difficult to be exactly assigned. Although the UL13 PKs are among the few known kinases with specificity for acidic substrates, they are quite distinct from cellular CKI and CKII families in terms of primary sequence.

TABLE 3

Effect of Various Protein Kinase Activators and Inhibitors on HSV-2 UL13 Protein Kinase, Protein Kinase CK I, and CK II

Activators or inhibitors	Percentage activity		
	CK I	CK II	UL13 PK
Spermine (0.5 m*M*)	115	83	120
(5 m*M*)	110	50	116
Protamine (100 μg/ml)	111	126	109
cAMP (10 μM)	99	107	100
$CaCl_2$ (1 m*M*) + Calmodulin (0.01 μM)	93	90	97
Poly-L-arginine (200 μg/ml)	98	144	99
Poly-L-lysine (200 μg/ml)	99	170	90
N-ethylmaleimide (10 m*M*)	52	65	52
H-9 (100 μM)	62	93	74
Staurosporin (20 μM)	101	98	76
Sphingosine (100 μg/ml)	100	94	103
ATP (0.2 m*M*)	49	34	40
(1 m*M*)	8	9	12
GTP (0.2 m*M*)	97	60	98
(1 m*M*)	90	22	86

Note. Reaction conditions are given under Materials and Methods.

DISCUSSION

The present study demonstrates that PK encoded by the UL13 gene of HSV-2 had distinct properties from those of cellular PKs and viral PK encoded by the US3 gene. When Vero cells were infected with HSV-1 and HSV-2, novel casein kinase activity was induced at the late stage of infection and the most striking induction was observed in the nuclear fractions of HSV-2-infected cells. We therefore tried to purify the virus-induced PK from the nuclear extracts of HSV-2-infected cells harvested at 20 hr postinfection. The purified preparation contained a single major protein with an apparent molecular weight of 56 kDa which was specif-

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HSV1 UL13 ....R..... G....NL... ..RQ....D. ....V..NPH G.........  50
HSV2 UL13 MDESGRQRPA SHVAADISPQ GAHRRSFKAW LASYIHSLSR RASGRPSGPS  50

HSV1 UL13 LQ.A...RSS H...H..GL. ...R...... .M....H..A ...T..T...  100
HSV2 UL13 PRDGAVSGAR PGSRRRSSFR ERLCAGLSRW RLSRSSRRRS SPEAPGPAAK  100

HSV1 UL13 .N.......Q A.L.A...S. ....T.T..R ...S....I. ...H......  150
HSV2 UL13 LRRPPLRRSE TAMTSPPSPP SHILSLARIH KLCIPVFAVN PALRYTTLEI  150

HSV1 UL13 .......... ...D...... ........K. ........I. .......V..  200
HSV2 UL13 PGARSFGGSG GYGEVQLIRE HKLAVKTIRE KEWFAVELVA TLLVGECALR  200
                     I           II                  III

HSV1 UL13 A....N.... .A........ .......... .......... ...T.N...S  250
HSV2 UL13 GGRTHDIRGF ITPLGFSLQQ RQIVFPAYDM DLGKYIGQLA SLRATTPSVA  250
               IV                      V

HSV1 UL13 ....Q...E. .........T .......... ..I....... ..........  300
HSV2 UL13 TALHHCFTDL ARAVVFLNTR CGISHLDIKC ANVLVMLRSD AVSLRRAVLA  300
                                        VI

HSV1 UL13 .......... ...A...... .....K...M ....T..... ..........  350
HSV2 UL13 DFSLVTLNSN STISRGQFCL QEPDLESPRG FGMPAALTTA NFHTLVGHGY  350
          VII

HSV1 UL13 .......... ........T. HR........ .......... ...V......  400
HSV2 UL13 NQPPELLVKY LNNERAEFNN RPLKHDVGLA VDLYALGQTL LELLVSVYVA  400
               VIII                          IX

HSV1 UL13 .........F .......... .....L.... .......... ..........  450
HSV2 UL13 PSLGVPVTRV PGYQYFNNQL SPDFAVALLA YRCVLHPALF VNSAETNTHG  450
                                    X

HSV1 UL13 .......... .......... ...DR..... H....I.... A.....K...  500
HSV2 UL13 LAYDVPEGIR RHLRNPKIRR AFTEQCINYQ RTHKAVLSSV SLPPELRPLL  500

HSV1 UL13 ........T. .C...A..      518
HSV2 UL13 VLVSRLCHAN PAARHSLS      518
               XI
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FIG. 7. The predicted amino acid sequence of the HSV-2 UL13 PK. The sequence is presented in the single-letter code and amino acids are numbered at the left end of each line. The amino acid which differed from that presented in HSV-1 strain KOS are indicated by aristrics. Underlined residues correspond to readily identifiable conserved subdomains of protein kinases (Hanks *et al.*, 1988). Subdomains are numbered below the sequence.

ically reacted with the antiserum against the His · Tag-UL13 fusion protein. Previously, Cunningham *et al.* (1992) have also reported the induction of a nuclear PK by HSV-1. In their study, however, the PK activity in nuclear extracts rapidly increases at the early step of infection and reached the peak by 6 hr postinfection. The PK efficiently phosphorylates an endogenous protein with a molecular weight of 57 kDa, which was found to be the UL 13 product itself. The enzyme is able to also phosphorylate exogenous casein, can use ATP or GTP as a phosphate donor, is stimulated by high concentrations of NaCl (1.5 *M*), and is resistant to inhibition by heparin. The UL13 PK purified by us was different from that enzyme in some important properties. Our enzyme was sensitive to NaCl, that is, the addition of 1 *M* NaCl inhibited the PK activity by more than 80%. In addition, the PK did not appear to utilize GTP as a phosphate donor. The reason for these differences remained unknown, but a possible explanation is that we used a purified preparation and exogenous casein as a substrate to characterize the enzyme while previous researchers studied endogenous protein phosphorylation using nuclear extracts of infected cells. In their case, it may be difficult to rule out that cellular PKs are responsible for the phosphorylation of endogenous proteins. Another possibility is that we used the enzyme purified from HSV-2-infected cells. It is known that some HSV-2-induced enzymes are different from their HSV-1 counterparts in some respects. For example, HSV-1-specific thymidine kinase has dTMP kinase activity while the HSV-2 enzyme does not (Fyfe, 1982); HSV-2 encodes a deoxyuridine triphosphate which, in contrast to the type 1 enzyme, fractionates into the cytoplasma (Wohlrab *et al.,* 1982). However, the properties of partially purified HSV-1 UL13 PK was similar to those of the HSV-2 enzyme although the HSV-1 preparation used was much lower in specific activity than that of the HSV-2 preparation (data not shown). It seems unlikely that the differences are attributed to difference of HSV types.

When the properties of the HSV-2 UL13 PK were compared with those of cellular CK I and CK II, the HSV PK resembled CK I rather than CK II. The UL 13 PK, like CK I, were insensitive to heparin, a potent inhibitor of CK II, did not utilize GTP, and were not stimulated by polyarginine and polylysine. However, the viral PK was much more resistant to a specific CK I inhibitor, CK I-7, and was more sensitive to quercetin. There was also a significant difference among these PKs in salt sensitivity. These data would be useful to differentiate the UL13 PK activity from multifunctional and ubiquitous CK I and CK II. As regards the substrate specificity, we found that the UL13 PK preferred α-casein to β- and κ-caseins and phosvitin. Most Ser/Thr protein kinases including CK I and CK II have been shown to recognize consensus sequences specified by basic or acidic residues situated in the proximity of the target residues and the overall acidic nature of the substrates of casein kinases is echoed by the presence of acidic residues in the vicinity of the target residues (Kuenzel *et al.,* 1987; Marin *et al.,* 1986). For these acidtropic protein kinases, it is becoming apparent that phosphate groups are important elements of substrate recognition, in some cases more critical than the directly coded acidic residues Asp and Glu (Flotow *et al.,* 1990). At present we cannot yet define the substrate determinants for the HSV UL13 PK. It will be interesting to determine whether the UL13 PK requires prior phosphorylation of substrates.

The UL 13 proteins of HSV-2 consisted of 528 amino acids and had the same size as the HSV-1 counterpart. The amino acid homology between the HSV-1 and HSV-2 PKs was 85.9%, which was a relatively high value among that of the other known proteins encoded by HSV-1 and HSV-2, for example, such values for the amino acid differences for thymidine kinase, glycoprotein C, alkaline nuclease, and DNA polymerase are 73, 73, 81, and 90.9%. A large number of protein kinase-related genes have been sequenced and analyzed on their amino acid sequence patterns. Hanks *et al.* (1988) have reported the identification of 11 conserved regions in the catalytic domains of PKs on the basis of an alignment of 65 different members of the PK family. The UL13 PKs have many of the sequence features that are highly conserved in all PKs and match the serine/threonine kinase pattern. The motif, Gly-X-Gly-X-X-Gly, recognized in many nucleotide-binding protein, was found in subdomain I and a lysine residue at position 176, which has been suggested to be directly involved in the phosphotransfer reaction, was found in subdomain II. However, the consensus triplets Asp-Phe-Gly in subdomain VII and Ala-Pro-Glu in subdomain VIII were not conserved in the HSV UL13 PKs although the Asp-Phe-Ser and Pro-Pro-Glu aligned at the locations may fit the consensus triplets, respectively. The Ala-Pro-Glu consensus is very important in catalysis (Hanks *et al.,* 1988) and mutagenesis studies have shown that each residue in this triplet is essential for activity of v-Src (Bryant and Parsons, 1983). Although the UL13 PK has Pro instead of Ala, the function of this subdomain is considered to be unchanged. It would be interesting to note that protein kinases belonging to the CK I family also lack the Ala-Pro-Glu motif in subdomain VIII (Graves *et al.,* 1993). Subdomain IX contains a well-conserved short stretch which includes the nearly invariant residues corresponding to Asp and Gly and was readily recognized in the UL13, while it was difficult to localize the subdomain X and XI. The latter must contain an invariant Arg near the catalytic domain carboxy terminus. The most possible candidate may be the arginine at position 505. Since the UL13 gene product not only acts as protein kinase

but also is a structural component of the virion, the conservation and divergence of the amino acid sequences of the UL13 PKs should be considered from both points of view.

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